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14. ABSTRACT The U.S. Navy maintains and deploys approximately 70 bottlenose dolphins for military operations and research. Health maintenance of these animals is critical to the success of the Navy's mission. Functional genomic approaches offer the potential to complement traditional methods of health assessment with rapid, sensitive and highly discriminative tests for health, infection, and exposure to chemical, biological and physical stress. To this end we have initialized development of a dolphin gene microarray in order to evaluate its utility as a transcriptomic biosensor in the health assessment of dolphins. To this end, normalized dolphin cDNA libraries have been generated from stimulated and unstimulated dolphin peripheral blood leukocytes. Expressed sequence tags (ESTs) have been collected and sequenced. Target genes (both immune response and stress-related) have been amplified and segments cloned for subsequent microarray development as well as for cloning of the full-length genes. The immunoglobulin genes were further studied and characterized at the molecular level. These valuable molecular tools will not only help in characterization of the dolphin immune system, but will be utilized in the development of a dolphin gene microarray to use as a transcriptomic biosensor in the health assessment of Navy and wild dolphins.

15. SUBJECT TERMS

Functional Genomics, Dolphin, Expressed Sequence Tags, Microarray, Transcript Profiling, Immune System

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FINAL REPORT

GRANT #: N00014-02-1-0386

PRINCIPAL INVESTIGATOR: Tracy Romano, PhD
CO-PRINCIPAL INVESTIGATOR: Greg Warr, PhD

INSTITUTION: Medical University of South Carolina

GRANT TITLE: A Functional Genomics Approach to Understanding and Evaluating Health in Navy Dolphins

AWARD PERIOD: 1 April 2002 – 31 May 2004

OBJECTIVES:
The long-term overall objective of this project is to develop and validate functional genomics-based diagnostic tools for assessing dolphin health. Expressed sequence tags (ESTs) specific to the dolphin immune system will be collected and used in transcript profiling to monitor expression levels during health, illness, after exposure to stressors, and/or after vaccine administration.

The specific objectives during the duration of ONR support were:
1). To develop dolphin cDNA libraries from unstimulated and stimulated dolphin peripheral blood lymphocytes (PBL) 2). To normalize a dolphin cDNA library for EST collection 3). To collect, sequence, and identify ESTs specific to the immune system 4). To clone segments of immune-related genes for the microarray and for future cloning of full-length genes 5). To initiate evaluation of available microarrays from other species for use with dolphin transcript profiling 6). To initiate development of a dolphin-specific microarray

APPROACH :
Dolphin normalized cDNA libraries will be constructed from both stimulated (Interleukin-2, T cell (Concanavalin A) and B cell (Lipopolysaccharide) dependent mitogens) and unstimulated dolphin PBL. ESTs will be collected from the normalized cDNA library and sequenced utilizing automatic DNA sequencing. Specific immune-related genes will be cloned by screening the library with primers from conserved sequences of aligned homologues from other closely related species such as the artiodactyls and polymerase chain reaction (PCR) technology. Once an adequate number of dolphin sequences have been collected, they will be amplified as cDNA, purified and printed on glass microarray slides. A swine microarray will be tested in parallel with the dolphin microarray. Ultimately, the microarrays will be utilized in transcript profiling experiments with cDNA derived from Navy dolphins of known health status. Bioinformatics will be used to analyze the information from the microarrays and for relating transcriptional signatures to the health of Navy dolphins.

ACCOMPLISHMENTS

ESTs were collected from a cDNA Lambda Zap library created from LPS stimulated dolphin PBL and analyzed via sequence comparison to available sequences in GenBank through BlastX and BlastN searches. Fifty-seven initial ESTs were collected and analyzed. The ESTs were found to be valid sequences of good length, some of which showed sequence homology to transcription factors and immune-related genes such as MHC class I, Interleukin 18, and a TNF-induced protein. A second Lambda Zap cDNA library was prepared from unstimulated dolphin PBL and PBL stimulated with LPS, ConA, and IL-2. The library yielded over 300,000 primary clones with insert sizes ranging from 0.6kb to >3kb (average 1.3 kb). Initial analysis of redundancy indicated a high percentage of hemoglobin α and β sequences. Despite several attempts to subtract the hemoglobin, the level of hemoglobin transcripts never fell below 12-15%. A total of approximately 700 clones were sequenced. To address the high redundancy, the library construction methodology was changed to a PCR based system. Two libraries were created utilizing this system from IL-2 and LPS stimulated blood cells respectively. Both libraries have been mined and approximately 35,000 clones isolated. An initial sequence analysis of the first 192 clones of the IL-2 library indicates an internal redundancy below 10%. To date, a total of 2592 ESTs have been sequenced from the IL-2 library and 192 from the LPS stimulated library. The IL-2 library sequences are in the process of being edited and annotated. For the LPS library, the internal redundancy is estimated to be about 15%. Basically all redundancy is caused by 3 genes; hemoglobin; 18S ribosomal gene; and IL-8 receptor. In order to reduce the redundancy, the library has been arrayed onto nylon filters and screened for the 3 genes. All positive clones are potentially eliminated in a rearraying procedure and additional clones are about to be sequenced.

Immunologically relevant dolphin genes and stress-related genes were isolated (Table 1) for construction of the dolphin microarray and for future cloning of the full-length genes. The immunoglobulin genes were further studied and characterized at the molecular level. The full sequence of dolphin Immunoglobulin M (IgM) heavy chain, including both the membrane bound and the secretory form was isolated and analyzed. The secretory form of IgM was isolated by screening a dolphin PBL cDNA library with a murine probe. A specific primer designed from exon 4 of IgM was used to amplify the transmembrane tail of the membrane-bound form of IgM (μTM). Detailed findings of the molecular characterization of dolphin IgM were published in *Developmental and Comparative Immunology* (Lundqvist, M.L., K.E. Kohlberg, H.A. Gefroh, P. Arnaud, D.L. Middleton, T.A. Romano, and G.W. Warr. 2002. Cloning of the IgM heavy chain of the bottlenose dolphin (*Tursiops truncatus*), and initial analysis of VH gene usage. Dev. Comp. Immunol., 26(6): p. 551-562).

Full-length sequences of dolphin IgA and two forms of IgG heavy chains (α and γ) have been generated by PCR amplification using degenerate primers of conserved regions from multiple species with reverse-transcribed dolphin spleen RNA as template. Subsequently, sequence specific primers were designed and then used in modified 3' and 5' RACE to obtain the full length sequence. The transmembrane form of γ was isolated by PCR amplification using primers designed from alignment of mouse and human transmembrane sequences in combination with a primer anchored in Cγ3. Southern blot analysis was performed on dolphin genomic DNA to assess the genomic organization of α and γ. In addition, a pig microarray was obtained (gift from Drs. Alan Mileham and Gary Evans of Sygen) in order to investigate the pig microarray as a

useful tool for gene expression analysis in cetaceans, given the close evolutionary relationship between cetaceans and artiodactyls. Future studies conducted in our laboratory will include investigation of the pig microarray as a useful tool for gene expression analysis in cetaceans, and continued development of the dolphin microarray.

Table 1: Immune and Stress-Related Genes of Dolphin cloned for the microarray

Igμ	**IL-1α, β**	**IL-5Rβ**	**TLR-1**
FcγRII	**IL-2**	**TNF–α**	**TLR-2**
MHCI	**IL-4**	**IFN-γ**	**TLR-3**
β-2 microglobulin	**IL-6**	**GM-CSF**	**TLR-4**
CD79a, b	**IL-8**	**TGF-β**	**IκBK-α**
Igγ1	**IL-10**	**RANTES**	**IκBK-β**
Igγ2	**IL-12**	**CCchemRg**	**PKC**
Igα	**IL-13**	**CYP1A1**	**STAT-4**
Igλ	**IL-16**	**AhR**	**STAT5B**
Lysozyme	**IL-17**	**ARNT**	**HSP-70**
IL-8R	**IL-18**	**MHC IIα/β**	**HSP-90**
TCRα	**STAT1**	**STAT6**	**NFkB**

CONCLUSIONS:

The molecular tools we have generated will allow for the continued progress towards the generation of a dolphin microarray and the validation of transcript profiling as a tool for assessing dolphin health. Moreover, the full-length Ig dolphin gene sequences we have obtained have allowed for comparative and evolutionary information on the dolphin immune system. Analysis of the available mammalian TM sequence alignments reveal that the dolphin μTM sequence has an unexpected amino acid shift in the well-conserved CART motif. Due to the conserved nature of the CART motif and its role in the immunoglobulin-CD79α/β interaction, the amino acid substitution in the dolphin CART motif may affect the efficiency of signal transduction by the dolphin membrane-bound IgM and warrants further investigation.

Analysis of dolphin γ sequences indicates the presence of two isotypes, γ-1 & γ-2 which differ mainly in the hinge region of the IgG molecule. PCR evidence from genomic DNA revealed a putative third isotype. The third isotype is currently under investigation. Genomic Southern blot analysis indicates the γ genes are most likely present in single copy and are closely linked. Genomic Southern blot indicates α genes may be present in up to five copies, but cDNA sequence data identified only a single α sequence, suggesting that the additional bands observed by Southern blot represent pseudo α genes. The deduced amino acid sequences of the dolphin immunoglobulins show greatest similarity to α and γ of evolutionarily-related artiodactyl species (pig, sheep, and cow). The dolphin γ-TM sequence shows similarity to other mammalian γ-TM sequences and does not show the Ser to Gly substitution of the CART motif found in dolphin μ-TM. Molecular cloning and characterization of these and other immune relevant genes of the dolphin will provide valuable information in regards to the evolution of the immune system as

well as contribute to human and veterinary medicine while allowing for investigation of utilizing a dolphin microarray and transcript profiling for health assessment in Navy and wild bottlenose dolphins.

SIGNIFICANCE:
The U.S. Navy maintains and deploys approximately 70 bottlenose dolphins for military operations and research. Health maintenance of these animals is critical to the success of the Navy's mission. The dolphin immune system is not well understood and the necessary tools for assessing dolphin health by traditional methods are under development. Functional genomic approaches offer the potential to complement traditional methods of health assessment with rapid, sensitive and highly discriminative tests for health, infection, and exposure to chemical, biological and physical stress. Functional genomics methodologies will also be applicable to wild cetaceans including stranded, threatened and endangered species. Comparison of dolphin gene sequences (e.g. immunoglobulin genes) with other mammalian species will contribute to information on the evolution of the immune system as well as human and veterinary medicine.

AWARD INFORMATION:
The graduate student funded by this proposal, Holly Gefroh, graduated from the Medical University of South Carolina with a Master's degree in May, 2004.

The P.I. was promoted to Vice President of Research & Veterinary Services at the Mystic Aquarium & Institute for Exploration and moved her laboratory from San Diego to Mystic, CT in May, 2004.

PUBLICATIONS AND ABSTRACTS:
Lundqvist, M.L., K.E. Kohlberg, H.A. Gefroh, P. Arnaud, D.L. Middleton, T.A. Romano, and G.W. Warr, Cloning of the IgM heavy chain of the bottlenose dolphin (Tursiops truncatus), and initial analysis of VH gene usage. *Developmental and Comparative Immunology*, 2002. 26(6): p. 551-562.

Gefroh, H.A., M.L. Lundqvist, G.W. Warr and T.A. Romano. Characterization of immunoglobulin μ, γ, and α heavy chains in the Atlantic bottlenose dolphin, *Tursiops truncatus*. Abstract presented at: *Medical University of South Carolina's Annual Student Research Day* November 2002, Charleston, SC.

Gefroh, H.A., M.L. Lundqvist, G.W. Warr, T.A. Romano. Characterization of immunoglobulin μ, γ, and α heavy chains in the Atlantic bottlenose dolphin, *Tursiops truncatus*. Oral presentation at the 9th International Congress of ISDCI, June 29 – July 4, 2003 St Andrews, Scotland, UK, p.124.

Gefroh, H.A., A. Mancia, M.L. Lundqvist, G.W. Warr, T.A. Romano. Dolphins and the Marine Environment: Testing the Sentinel Hypothesis with Functional Genomics. Abstract presented at: *Medical University of South Carolina's Annual Student Research Day* November 2003, Charleston, SC.

Gefroh, Holly. 2004. Molecular Characterization of Immunoglobulins A, G, and M Heavy Chains in the Atlantic Bottlenose Dolphin, *Tursiops truncatus*. 2004. *Master of Science Dissertation*. Medical University of South Carolina, Charleston, SC.

Gefroh, H.A., T.A. Romano, G.W. Warr, and M.L. Lundquist. IgA and IgG Heavy Chain Genes of the Atlantic Bottlenose Dolphin, *Tursiops truncatus*: Structure, expression and diversity. *In Preparation*.